FORM PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

ATTORNEY'S DOCKET NUMBER: SCH 1743

U.S APPLICATION NO (If known, see 37 CFR §1 5): 09/509608

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/06134	28 September 1998	30 September 1997

TITLE OF INVENTION

PROCESS FOR THE PRODUCTION OF ERGOSTEROL AND ITS INTERMEDIATE PRODUCTS USING RECOMBINANT YEASTS

APPLICANT(S) FOR DO/EO/US

WEBER, Alfred, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3))
   a ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. §371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 13. to 19. below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 C.F.R. §§1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. §§3.28 and 3.31 is included.
15. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

422 Rec'd PCT/PTO 30 MAR 2000

U.S. APPLICATION NO. (if known, see 37 CFR §1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/509608	PCT/EP98/06134	SCH 1743

17. ☒ The following fees are submitted:		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR §1.492 (a) (1) - (5)):			
Search Report has been prepared by the EPO or JPO	$840.00		
International preliminary examination fee paid to USPTO (37 CFR §1.482)	$670.00		
No international preliminary examination fee paid to USPTO (37 CFR §1.482) but international search fee paid to USPTO (37 CFR §1.445(a)(2))	$760.00		
Neither international preliminary examination fee (37 CFR §1.482) nor international search fee (37 CFR §1.445(a)(2)) paid to USPTO	$970.00		
International preliminary examination fee paid to USPTO (37 CFR §1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(e)).		$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	26 - 20 =	6	x $ 18.00	$108.00	
Independent claims	8 - 3 =	5	x $ 78.00	$390.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 260.00		
TOTAL OF ABOVE CALCULATIONS =					
Reduction of ½ for filing by small entity, if applicable. A Verified Small Entity Statement must also be filed (Note 37 C.F.R. §§1.9, 1.27, 1.28).					
SUBTOTAL =				$1,468.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. §1.492(f)).					
TOTAL NATIONAL FEE =				$1,468.00	
Fee for recording the enclosed assignment (37 C.F.R. §1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§3.28, 3.31). $40.00 per property.					
TOTAL FEES ENCLOSED =				$1,468.00	
				Amount to be refunded:	
				charged:	

a. ☒ A check in the amount of $1,468.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 13-3402 in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-3402. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 C.F.R. §§1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. §1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Filed: March 30, 2000

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IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No.	:	PCT/EP98/06134
International Filing Date	:	28 September 1998
Priority Date(s) Claimed	:	30 September 1997
Applicant(s) (DO/EO/US)	:	WEBER, Alfred, et al.

Title: PROCESS FOR THE PRODUCTION OF ERGOSTEROL AND ITS INTERMEDIATE PRODUCTS USING RECOMBINANT YEASTS

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The claims of the above-identified International Application were amended under Articles 19 and/or 34 of the PCT. It is requested that examination in the U.S. National Phase be based on the claims as originally filed under the PCT and this Preliminary Amendment is based on the original claims.

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

IN THE CLAIMS:

Claim 4: Please rewrite the claim, as follows:

4. (Amended) Process for the production of ergosterol and its intermediate products according to claim 1, wherein the genes [that are mentioned in claim 1 under a), those in claim 2 under a-i) to a-vii) and those in claim 3 under a-ii), a-iii) and a-v)] in each case with the plasmids are first introduced independently of one another into microorganisms of the same species, and fermentation into ergosterol is performed with them together and the ergosterol that is thus obtained is extracted from the cells, analyzed and purified using column chromatography and isolated.

Claims 5, 6, 7 and 8, line 1: Change "claims 1 to 4" to -- claim 1 --.

Claims 15, 16 and 17, line 1: Change "claims 12 to 14" to -- claim 12 --.

Claim 21, lines 1 and 2: Change "claims 18 to 20" to -- claim 18 --.

Claim 23, line 2: Change "claims 18 to 20" to -- claim 18 --.

Claims 25 and 26, line 1: Change "claims 23 and 24" to -- claim 23 --.

Remarks

The purpose of this Preliminary Amendment is to eliminate the multiple dependency of the claims in order to avoid the additional fee.

Respectfully submitted,

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Process for the Production of Ergosterol and its Intermediate Products using Recombinant Yeasts

This invention relates to a process for the production of ergosterol and its intermediate products using recombinant yeasts and plasmids for the transformation of yeasts.

Ergosterol is the end product of sterol synthesis in yeasts and fungi. The economic importance of this compound lies, on the one hand, in obtaining vitamin D_2 from ergosterol with UV irradiation, and, on the other hand, in obtaining steroid hormones with biotransformation, starting from ergosterol. Squalene is used as a synthesis component for the synthesis of terpenes. In hydrogenated form, it is used as squalene in dermatology and cosmetics and in various derivatives as components of skin and hair cleansers. Also of economic importance are the intermediate products of the ergosterol metabolic process. Farnesol, geraniol and squalene can be named as most important here. In addition, sterols, such as, e.g., zymosterol and lanosterol, can be used economically, whereby lanosterol is pivotal in terms of crude and synthesis for the chemical synthesis of saponins and steroid hormones. Because of its good skin penetration and spreading properties, lanosterol is used as an emulsion adjuvant and active ingredient for skin creams.

The genes of the ergosterol metabolism in yeast are largely known and cloned, e.g., the HMG-CoA reductase (**HMG1**) (Basson et al. (1988)), the squalene synthetase (**ERG9**) (Fegueur et al. (1991)), the acyl-CoA: sterol-acyl transferase (**SAT1**) (Yu et al. (1996)), and the squalene epoxidase (**ERG1**) (Jandrositz et al. (1991)). Squalene synthetase catalyzes the reaction of farnesyl pyrophosphate on presqualene pyrophosphate to squalene. The reaction mechanisms of sterol-acyl transferase are not fully determined. An over-expression of genes of these above-mentioned enzymes was already attempted, but it did not result in any significant increase in the amount of ergosterol. In the case of the **HMG1** over-expression, the overproduction of squalene was described; moreover, additional mutations were introduced to interrupt the route following squalene (EP-0 486 290).

The overproduction of geraniol and farnesol was also described, but here no over-expression of genes of the ergosterol metabolism took place, rather an interruption of the reaction process as regards geraniol and farnesol formation (EP-0313 465).

Specific inhibitors of the ergosterol biosynthesis can also result in the accumulation of larger amounts of certain intermediate products, e.g., allylamines, which prevent the conversion of squalene into squalene epoxide. As a result, large amounts (up to 600 times the normal level) of squalene are accumulated (Jandrositz et al., (1991)).

Although the use of inhibitors leads to a major accumulation of, e.g., squalene, the addition of these substances may yet turn out to be disadvantageous since only small amounts exert the same

action in the organism, so that a production of the products of ergosterol biosynthesis in the process of overproduction is advantageous.

The object of this invention is to synthesize a microbiological process for the production of ergosterol and its intermediate products, the microorganisms that are necessary for this purpose, such as yeast strains, the increased amounts of ergosterol or intermediate products that are necessary for this purpose, and to prepare the plasmids that are necessary for the transformation of the yeast strain.

It was now found that the amount of ergosterol and its intermediate products can be increased, if the genes of HMG1 (Basson et al., (1988)), **ERG9** (Fegueur et al., (1991)), Current Genetics 20: 365-372), **SAT1** (Yu et al., (1996)) and **ERG1** (Jandrositz et al. (1991)) are introduced in altered form into microorganisms such as, e.g., yeasts, whereby the genes are located either individually on a plasmid or in combination on one or more plasmids and can be brought to the host simultaneously or in succession.

The subject of this invention is thus a process that is characterized in that

a) first a plasmid is designed, into which several suitable genes of the ergosterol metabolic process are inserted in altered form,

or

b) first plasmids are designed, into which in each case one of the genes of the ergosterol metabolic process is inserted in altered form,

c) microorganisms are transformed with the thus produced plasmids, whereby the microorganisms are transformed with a plasmid under a) or they are transformed simultaneously or in succession with several plasmids under b),

d) fermentation into ergosterol is performed with the thus produced microorganisms,

e) after fermentation has ended, the ergosterol and its intermediate products are extracted from the cells and analyzed, and finally,

f) the thus obtained ergosterol and its intermediate products are purified using column chromatography and isolated.

The subject of this invention is especially a process which is characterized in that

a-i) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

ii) the gene of squalene synthetase (**ERG9**),

iii) the gene of Acyl-CoA: sterol-acyl transferase (**SAT1**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-ii) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

and

ii) the gene of squalene synthetase (**ERG9**),

or

a-iii) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

and

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**),

or

a-iv) first a plasmid is designed, into which the following genes are inserted:

i) the gene of the HMG-Co-A-reductase (t-**HMG**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-v) first a plasmid is designed, into which the following genes are inserted:

ii) the gene of squalene synthetase (**ERG9**),

and

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**)

or

a-vi) first a plasmid is designed, into which the following genes are inserted:

ii) the gene of squalene synthetase (**ERG9**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-vii) first a plasmid is designed, into which the following genes are inserted:

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

b) first plasmids are designed, into which in each case one of the genes that is mentioned under a-i) is inserted,

and

c) microorganisms are transformed with the thus produced plasmids, whereby the microorganisms are transformed with a plasmid under a-i) to a-vii), or they are transformed simultaneously or in succession with several plasmids under b),

d) fermentation into ergosterol is performed with the thus produced microorganisms,

e) after fermentation has ended, the ergosterol and its intermediate products are extracted from the cells and analyzed, and finally

f) the thus obtained ergosterol and its intermediate products are purified using column chromatography and isolated.

In addition, the gene of squalene epoxidase (**ERG1**) can be inserted into the plasmids that are cited under a-ii), a-iii) and a-v), and in addition, the gene of acyl-CoA: sterol-acyl transferase (**SAT1**) can be inserted into the plasmid that is cited under a-ii). These plasmids are also subjects of this invention.

Intermediate products are defined as squalene, farnesol, geraniol, lanosterol, zymosterol, 4,4-dimethylzymosterol, 4-methylzymosterol, ergost-7-enol and ergosta-5,7-dienol, especially sterols with 5,7-diene structure.

The plasmids that are used are preferably the plasmid YEpH2, which contains the average **ADH**-promoter, t-HMG (altered variant of **HMG1**) and the **TRP**-terminator (see Fig. 1), the plasmid YDpUHK3, which contains the average **ADH**-promoter, t-HMG (altered variant of **HMG1**) and the **TRP**-terminator, the gene for the kanamycin resistance and the **ura3** gene (see Fig. 2) and the plasmid pADL-SAT1, which contains the **SAT1** gene and the **LEU2** gene of YEp13.

These plasmids and their use for the production of ergosterol and its intermediate products, such as squalene, farnesol, geraniol, lanosterol, zymosterol, 4,4-dimethylzymosterol, 4-methylzymosterol, ergost-7-enol and ergosta-5,7-dienol, especially sterols with 5,7-diene structure, are also subjects of this invention.

As a host for the introduction of plasmids according to the invention, in principle all microorganisms, especially yeasts, are suitable.

The species **S. cerevisiae**, especially the strain **S. cerevisiae** AH22, is preferred.

The subject of this invention is also the yeast strain **S. cerevisiae** AH22, which contains one or more of the genes that are mentioned in the process under a-i).

The subject of this invention is also the yeast strain **S. cerevisiae** AH22, which contains the plasmid pADL-SAT1.

In addition, the combined transformation of microorganisms with the plasmids pADL-SAT1 and YDpUHK3, especially yeasts such as **S. cerevisiae** AH22, is preferred.

Viewed overall, the flow in the ergosterol metabolic process is affected as follows:

The flow in the direction of ergosterol is maximized by the activity of several bottle-neck enzymes being intensified simultaneously. In this case, various enzymes play a decisive role, whereby the combination of deregulation or over-expression provides the decisive breakthrough for increasing the ergosterol yield. As combinations, the enzymes or their genes **HMG1** (Basson et al., (1988)), **ERG9** (Fegueur et al., (1991)), acyl-CoA: sterol-acyl transferase (**SAT1**) (Yu et al. (1996)) and/or squalene epoxidase (ERG1) (Jandrositz et al. (1991)) are introduced into a yeast strain in **altered** form, whereby the genes are introduced with one or more plasmids, whereby the DNA sequences are

contained either individually or in combination in the plasmid(s).

In the case of gene **HMG1**, "altered" means that of the corresponding genes, only the catalytic area is expressed without the membrane-bound domains. This alteration was already described (EP-0486 290). The purpose of the alteration of **HMG1** is to prevent the feedback regulation by intermediates of ergosterol biosynthesis. Both **HMG1** and the two other above-mentioned genes are removed in the same way from the transcriptional regulation. To this end, the promoter of the genes is replaced by the "average" **ADH1**-promoter. This promoter fragment of the **ADH1**-promoter shows an approximately constitutive expression (Ruohonen et al., (1995)), so that the transcriptional regulation no longer proceeds via intermediates of the ergosterol biosynthesis.

The products that are produced in the over-expression can be used in biotransformations or other chemical and therapeutic purposes, e.g., obtaining vitamin D_2 from ergosterol via UV irradiation, and obtaining steroid hormones via biotransformation starting from ergosterol.

Subjects of this invention are also microorganisms, especially yeast strains, which can produce an increased amount of ergosterol and ergosterol in combination with increased amounts of squalene by over-expression of the genes that are mentioned in the process under a-i).

Preferred is an altered variant of the gene **HMG1**, in which only the catalytic area is expressed without the membrane-bound domain. This alteration is described (EP-0486 290).

A subject of this invention is also a process for the production of ergosterol and its intermediate products, which is characterized in that the genes that are mentioned in the process under a), especially the genes that are mentioned in the processes under a-i to a-vii) (two-, three-, and four-fold gene combinations) in each case with the plasmids are first introduced independently of one another into microorganisms of the same species, and fermentation into ergosterol is performed with them together, and the ergosterol that is thus obtained is extracted from the cells, analyzed and purified using column chromatography and isolated.

Subjects of this invention are also expression cassettes, comprising the average **ADH**-promoter, the **t-HMG** gene, the **TRP**-terminator, and the **SAT1**-gene with the average **ADH**-promoter and the **TRP**-terminator and expression cassettes, comprising the average **ADH**-promoter, the **t-HMG** gene, the **TRP**-terminator, the **SAT1** gene with the average **ADH**-promoter and the **TRP**-terminator, and the **ERG9**-gene with the average **ADH**-promoter and the **TRP**-terminator.

A subject of this invention is also a combination of expression cassettes, whereby the combination consists of

a) a first expression cassette, in which the **ADH**-promoter, the **t-HMG** gene and the **TRP**-terminator are located,

b) a second expression cassette, in which the **ADH**-promoter, the **SAT-1** gene and the **TRP**-terminator are located,

and

c) a third expression cassette, in which the **ADH**-promoter and the **ERG9**-gene with the **TRP**-terminator are located.

The subject of this invention is also the use of these expression cassettes for the transformation of microorganisms, which are used in the fermentation into ergosterol, whereby the microorganisms are preferably yeasts.

Microorganisms such as yeasts, which contain these expression cassettes, as well as their use in the fermentation into ergosterol and ergosterol intermediate products, are also subjects of the invention.

The following examples are used for the explanation with respect to the implementation of the processes that are necessary for the embodiments:

1. Restriction

The restriction of plasmids (1 to 10 μg) was performed in 30 μl batches. To this end, the DNA was taken up in 24 μl of H_2O, and mixed with 3 μl of the corresponding buffer, 1 μl of RSA (bovine serum albumin) and 2 μl of enzyme. The enzyme concentration was 1 unit/μl or 5 units/μl depending on the amount of DNA. In some cases, 1 μl more of RNase was added to the batch to degrade the tRNA. The restriction batch was incubated for two hours at 37°C. The restriction was controlled with a minigel.

2. Gel Electrophoreses

The gel electrophoreses were performed in minigel or wide-minigel equipment. The minigels (about 20 ml, 8 bags) and the wide-minigels (50 ml, 15 or 30 bags) consisted of 1% agarose in TAE. 1 x TAE was used as a mobile buffer. The samples (10 µl) were mixed with 3 µl of stopper solution and applied. I-DNA cut with **Hind**III was used as a standard (bands at: 23.1 kb; 9.4 kb; 6.6 kb; 4.4 kb; 2.3 kb; 2.0 kb; 0.6 kb). For separation, a voltage of 80 V for 45 to 60 minutes was prepared. Then, the gel was stained in ethidium bromide solution and held under UV light with video-documentation system INTAS or photographed with an orange filter.

3. Gel Elution

The desired fragments were isolated using gel elution. The restriction preparation was applied in several bags of a minigel and separated. Only λ-**Hind**III and a "sacrifice trace" were stained in ethidium bromide solution, viewed under UV light, and the desired fragment was labeled. As a result, DNA was prevented from damaging the residual bags by the ethidium bromide and the UV light. By aligning the stained and unstained gel pieces, the desired fragment from the unstained gel piece could be cut out based on the labeling. The agarose piece with the fragment to be isolated was added in a dialysis tube, sealed free of air bubbles with a little TAE buffer and placed in the BioRad-minigel apparatus. The mobile buffer consisted of 1 x TAE, and the voltage was 100 V for 40 minutes. Then, the flow polarity was

varied for 2 minutes to loosen the DNA adhering to the dialysis tube. The buffer that contains the DNA fragments of the dialysis tube was moved into the reaction vessel and thus performed an ethanol precipitation. To this end, 1/10 volume of 3 M sodium acetate, tRNA (1 µl per 50 µl of solution) and 2.5 times the volume of ice-cold 96% ethanol were added to the DNA solution. The batch was incubated for 30 minutes at -20°C and then centrifuged off at 12,000 rpm for 30 minutes at 4°C. The DNA pellet was dried and taken up in 10 to 50 µl of H_2O (depending on the amount of DNA).

4. Klenow Treatment

Projecting ends of DNA fragments are made up by the Klenow treatment, so that "blunt ends" result. Per 1 µg of DNA, the following batch was pipetted together:

DNA-pellet +11 µl of H_2O
+ 1.5 µl of 10 x Klenow buffer
+ 1 µl of 0.1M DTT
+ 1 µl of nucleotides (dNTP 2 mmol)
+ 1 µl of Klenow-polymerase (1 unit/µl)

In this case, the DNA should be derived from an ethanol precipitation to prevent contaminants from inhibiting the Klenow-polymerase. Incubation was carried out for 30 minutes at 37°C, and then over another 5 minutes at 70°C the reaction was halted. The DNA was obtained from the batch by an ethanol precipitation and taken up in 10 µl of H_2O.

5. Ligation

The DNA fragments that were to be ligated were combined. The end volume of 13.1 µl contained about 0.5 µg of DNA with a vector-insert ratio of 1:5. The sample was incubated for 45 seconds at 70°C, cooled to room temperature (about 3 minutes) and then incubated on ice for 10 minutes. Then, the ligation buffers were added: 2.6 µl of 500 mmol TrisHCl, pH 7.5, and 1.3 µl of 100 mmol $MgCl_2$, and they were incubated on ice for another 10 minutes. After 1 µl of 500 mmol DTT and 1 µl of 10 mmol ATP were added, 1 µl of ligase (1 unit/µl) was added on ice for another 10 minutes. The entire treatment should be carried out with as little shaking as possible so as to keep adjacent DNA ends from reseparating. The ligation was carried out overnight at 14°C.

6. E. coli Transformation

Component Escherichia coli (E. coli) NM522 cells were transformed with the DNA of the ligation preparation. As a positive control, a batch was supplied with 50 ng of the pScL3 plasmid, and as a null control, a batch was supplied without DNA. For each transformation preparation, 100 µl of 8% PEG solution, 10 µl of DNA and 200 µl of competent cells (**E. coli** NM522) were pipetted into a tabletop centrifuging tube. The batches were put on ice for 30 minutes and shaken intermittently. Then, thermal shock took place: 1 minute at 42°C. For regeneration, 1 ml of LB-medium was added to the cells and incubated on a shaker for 90 minutes at 37°C. 100 µl each of the undiluted batches, a 1:10

dilution and a 1:100 dilution were flattened out on LB + ampicillin plates and incubated overnight at 37°C.

7. Plasmid Isolation from E. Coli (Miniprep)

E. coli colonies were cultured overnight in 1.5 ml of LB + ampicillin medium in tabletop centrifuging tubes at 37°C and 120 rpm. The next day, the cells were centrifuged off for 5 minutes at 5000 rpm and 4°C, and the pellet was taken up in 50 µl of TE-buffer. Each batch was mixed with 100 µl of 0.2N NaoH, 1% SDS solution, mixed and put on ice for 5 minutes (lysis of the cells). Then, 400 µl of Na-acetate/NaCl solution (230 µl of H_2O, 130 µl of 3 M sodium acetate, and 40 µl of 5 M NaCl) was added, the batch was mixed and put on ice for another 15 minutes (protein precipitation). After 15 minutes of centrifuging at 11,000 rpm, the supernatant, which contains plasmid-DNA, was moved into an Eppendorf vessel. If the supernatant was not completely clear, it was centrifuged one more time. The supernatant was mixed with 360 µl of ice-cooled isopropanol and incubated for 30 minutes at -20°C (DNA precipitation). The DNA was centrifuged off (15 minutes, 12,000 rpm, 4°C), the supernatant was discarded, the pellet was washed in 100 µl of ice-cooled 96% ethanol, incubated for 15 minutes at -20°C and centrifuged off again (15 minutes, 12,000 rpm, 4°C). The pellet was dried in a speed vacuum and then taken up in 100 µl of H_2O. The plasmid-DNA was characterized by restriction analysis. To this end, 10 µl of each batch was restricted and cleaved by gel electrophoresis in a wide-minigel (see above).

8. Plasmid-Working-Up on E. coli (Maxiprep)

To isolate larger amounts of plasmid-DNA, the maxiprep method was performed. Two plungers with 100 ml of LB + ampicillin medium were inoculated with a colony or with 100 µl of a frozen culture, which carries the plasmid that is to be isolated, and it was incubated overnight at 37°C and 120 rpm. The next day the culture (200 ml) was moved into a GSA beaker and centrifuged for 10 minutes at 4000 rpm (2600 x g). The cell pellet was taken up in 6 ml of TE-buffer. To digest the cell wall, 1.2 ml of lysozyme solution (20 mg/ml of TE-buffer) was added, and it was incubated for 10 minutes at room temperature. Then, the lysis of the cells was carried out with 12 ml of 0.2N NaOH, 1% SDS solution and for another 5 minutes of incubation at room temperature. The proteins were precipitated by the addition of 9 ml of cooled 3 M sodium acetate solution (pH 4.8) and a 15-minute incubation on ice. After centrifuging (GSA: 13,000 rpm (27,500 x g), 20 minutes, 4°C), the supernatant, which contained the DNA, was moved into a new GSA beaker, and the DNA was precipitated with 15 ml of ice-cold isopropanol and an incubation of 30 minutes at -20°C. The DNA pellet was washed in 5 ml of ice-cooled ethanol and dried in air (about 30-60 minutes). Then, it was taken up in 1 ml of H_2O. An examination of the plasmid by restriction analysis took place. The concentration was determined by depositing dilutions on a minigel. To reduce the salt content, a 30-60 minute microdialysis was carried out (pore size 0.025 µm).

9. Yeast Transformation

For the yeast transformation, a pre-culture of the strain **Saccharomyces cerevisiae (S. cerevisiae)** AH22 was prepared. A plunger with 20 ml of YE-medium was inoculated with 100 µl of the frozen culture and incubated overnight at 28°C and 120 rpm. The main cultivation was carried out under identical conditions in a plunger with 100 ml of YE-medium, which was inoculated with 10 µl, 20 µl or 50 µl of the pre-culture.

9.1 Producing Competent Cells

The next day, the plungers were counted out using a Thoma chamber, and the procedure was continued with the plunger, which held 3-5 x 10^7 cells/ml. The cells were harvested by centrifuging (GSA: 5000 rpm (4000 x g), 10 minutes). The cell pellet was taken up in 10 ml of TE-buffer and divided into two tabletop centrifuging tubes (5 ml each). The cells were centrifuged off for 3 minutes at 6000 rpm and washed twice more with 5 ml of TE-buffer each. Then, the cell pellet was taken up in 330 µl of lithium acetate buffer per 10^9 cells, moved into a sterile 50 ml Erlenmeyer flask and shaken for one hour at 28°C. As a result, the cells were competent for transformation.

9.2 Transformation

For each transformation preparation, 15 µl of herring sperm DNA (10 mg/ml), 10 µl of DNA that is to be transformed (about 0.5 µg) and 330 µl of component cells were pipetted into a tabletop centrifuging tube and incubated for 30 minutes at 28°C (without

shaking!). Then, 700 µl of 50% PEG 6000 was added, and it was incubated for one additional hour at 28°C, without shaking. A thermal shock of 5 minutes at 42°C followed.

100 µl of the suspension was flattened out on the selection medium (YNB, Difco) to select leukine prototrophy. In the case of the selection on G418 resistance, a regeneration of the cells is carried out after the thermal shock (see under 9.3 Regeneration Phase).

9.3 Regeneration Phase

Since the selection marker is resistance to G418, the cells needed time for the expression of the resistance-gene. The transformation preparations were mixed with 4 ml of YE-medium and incubated overnight at 28°C in the shaker (120 rpm). The next day, the cells were centrifuged off (6,000 rpm, 3 minutes), taken up in 1 ml of YE-medium, and 100 µl or 200 µl was flattened out on YE + G418 plates. The plates were incubated for several days at 28°C.

10. Reaction conditions for the PCR

The reaction conditions for the polymerase chain reaction must be optimized for the individual case and are not necessarily valid for any batch. Thus, i.a., the amount of DNA used, the salt concentrations and the melting temperature can be varied. For our formulation of the problem, it has proven advantageous to combine the following substances in an Eppendorf cap, which was suitable for use in a thermocycler: 5 µl of super buffer, 8 µl

of dNTP's (0.625 μM each), 5'-primer, 3'-primer and 0.2 μg of matrix DNA, dissolved in enough water to yield a total volume of 50 μl for the PCR preparation, were added to 2 μl (-0.1 U) of Super Taq polymerase. The batch was briefly centrifuged off and covered with a drop of oil. Between 37 and 40 cycles were selected for amplification.

The embodiments below describe the production of the plasmids and yeast strains according to the invention as well as their use, without, however, limiting the invention to these examples.

Example 1

Expression of tHMG in S. cerevisiae AH22

The DNA sequence for tHMG (Basson et al., (1988)) was amplified by PCR from genomic DNA of **Saccharomyces cerevisiae** S288C (Mortimer and Johnston, (1986)) with use of standard methods. The primers that are used in this case are the DNA oligomer tHMG-5′ and tHMG-3′ (see Seq ID Nos. 1 and 2). The DNA-fragment that was obtained was introduced in cloning vector pUC19 (Yanisch-Perron et al., (1985)) after a Klenow treatment, and yielded vector pUC19-tHMG. After plasmid isolation and restriction of pUC 19-tHMG with endonucleases **Eco**R1 and **Bam**H1, the obtained fragment was introduced into yeast expression vector pPT2b (Lang and Looman, (1995)), which also was treated with **Eco**R1 and **Bam**H1. The plasmid pPT2b-tHMG that was produced contains the **ADH1**-promoter (Bennetzen and Hall, (1982)) and the **TRP1**-terminator (Tschumper and Carbon, (1980)), between which the **tHMG**-DNA fragment is found. A DNA section was isolated from vector pPT2b-tHMG via endonucleases **Eco**RV and **Nru**I, and said DNA section contains the so-called average **ADH1**-promoter, the **tHMG** and the **TRP1**-terminator. This DNA section was introduced into yeast vector YEp13 (Fischhoff et al., (1984)), which was treated with endonuclease **Sph**I and a DNA polymerase. The vector that is

thus produced, the YEpH2 (Fig. 1), was treated with the endonucleases **Eco**RV and **Nru**I. A DNA-fragment with the following areas was thus produced: a transcription-activating area from the tetracycline resistance gene (Sidhu and Bollon, (1990)), the average **ADH1**-promoter, the **tHMG** and the **TRP1**-terminator (expression cassette). This DNA-fragment was introduced into vector YDpU (Berben et al., (1991)), which was treated with **Stu**I. Vector YDpUH2/12 that was thus produced was treated with endonuclease **Sma**I and ligated with a DNA-sequence that codes for a kanamycin resistance (Webster and Dickson, (1983)). The construct that is produced (YDpUHK3, Fig. 2) was treated with **Eco**RV. The yeast strain **Saccharomyces cerevisiae** AH22 was transformed with this construct. The transformation of the yeast with a linearized vector, as it is in this example, results in a chromosomal integration of the total vector at the **URA3** gene locus. To eliminate the areas from the integrated vector that are not part of the expression cassette (**E. coli** origin, **E. coli**-ampicillin resistance gene, TEF-promoter and kanamycin resistance gene), transformed yeasts were subjected to a selection pressure by FOA selection (Boeke et al., (1987)) that promotes uracil-auxotrophic yeasts. The uracil-auxotrophic strain that is described in the selection bears the name AH22/tH3ura8 and has the **tHMG1**-expression cassette as chromosomal integration in the **URA3**-gene.

The yeast strain AH22/tH3ura8 and the starting strain AH22 were cultivated for 48 hours in YE at 28°C and 160 rpm in a flow spoiler plunger.

Cultivation conditions: Pre-culture WMVIII was prepared as follows: 20 ml of WMVIII + histidine (20 µg/ml) + uracil (20 µg/ml) were inoculated with 100 µl of frozen culture and incubated for 2 days at 28°C and 120 rpm (reciprocal motion). From the 20 ml of pre-culture, 100 ml of WMVIII + histidine (20 µg/ml) + uracil (20 µg/ml) were inoculated. For the main culture, 50 ml of YE (in a 250 ml flow spoiler plunger) with 1 x 10^9 cells was inoculated. The plungers were incubated at 160 rpm in a round shaker at 28°C for 48 hours. HMG-CoA-reductase activities were determined (according to Qureshi et al., (1981)), and produced the following values (see Table 1).

Table 1

	Specific HMG-CoA-reductase activity* (U/mg of protein)
AH22	3.99
AH22/tH3ura8	11.12

* A unit is defined as the reaction of 1 nmol of NADPH per minute in a milliliter reaction mixture. The measurement was carried out with total protein isolates.

The sterols were extracted (Parks et al., (1985)) and analyzed using gas chromatography. The following values were produced (see Table 2).

Table 2

	Squalene (% w/w)	Ergosterol (% w/w)
AH22	0.01794	1.639
AH22/tH3ura8	0.8361	1.7024

The values, in percent, relate to the dry weight of the yeast.

Example 2

Expression of SAT1 in S. cerevisiae AH22

The sequence for the acyl-CoA: sterol transferase (**SAT1;** Yang et al., (1996)) was obtained by, as described above, PCR from genomic DNA of **Saccharomyces cerevisiae** S288C. The primers used in this case are the DNA-oligomers SAT1-5′ and SAT1-3′ (see Seq ID Nos. 3 and 4). The DNA-fragment that was obtained was cloned in cloning vector pGEM-T (Mezei and Storts, (1994)), which resulted in vector pGEM-SAT1. By treatment of pGEM-SAT1 with **Eco**R1, a fragment was obtained that was cloned in yeast expression vector pADH1001, which also was treated with **Eco**R1. Vector pADH-SAT1 that was thus produced was treated with the endonuclease **Nru**I, and it was ligated with a fragment of YEp13, which contains the **LEU2**-gene.

Yeast expression vector pADL-SAT1 (Fig. 3), which was introduced into yeast strain AH22, was thus produced. The thus obtained strain AH22/pADL-SAT1 was incubated for 7 days in WMVIII

(Lang and Looman (1995)) in a minimal medium. Cultivation conditions: (For pre-culture, see above) Main culture: 50 ml of WMVIII + histidine (20 µg/ml) + uracil (20 µg/ml) of cultures (in a 250 ml flow spoiler plunger) were inoculated with 1 x 10^9 cells: the plungers were incubated at 160 rpm on a round shaker at 28°C for 7 days. The sterols formed were analyzed via gas chromatography (see Table 3).

Table 3

	Squalene (% w/w)	Ergosterol (% w/w)
AH22	n.d.	1.254
AH22/pADL-SAT1	n.d.	1.831

The values, in percent, relate to the dry weight of the yeast.

n.d.: indeterminate

Example 3

Combined Expression of the Shortened 3-Hydroxy-3-methylglutaryl-CoA-Reductase (tHMG) and the Acyl-CoA: Sterol-acyl Transferase (SAT1)

Example 3.1

Yeast strain AH22/tH3ura 8 was transformed with the **SAT1** expression vector pADL-SAT1, and yielded AH22/tH3ura8/pADL-SAT1.

This combined strain was cultivated for 7 days in WMVIII. The sterols were extracted (see above) and analyzed via gas chromatography. The following values were produced (see Table 4).

Table 4

	Squalene (% w/w)	Ergosterol (% w/w)
AH22/tH3ura8	1.602	3.798
AH22/tH3ura8/pADL-SAT1	1.049	5.540

The values, in percent, relate to the dry weight of the yeast.

Example 3.2

Yeast cultures were cultivated for 7 days in WMVIII, but different amounts of uracil were added to the cultures. Concentrations of 10, 20, 40 and 100 µg/ml of uracil were set in the medium. The ergosterol and the squalene amounts are at most in a supplementation of 20 µg/ml of uracil. The results are shown in Fig. 4.

It is shown that the ergosterol and squalene yield in strain AH22tH3ura8/pADL-SAT1 depends greatly on the amount of uracil that is added to cultivation medium WMVIII.

Example 3.3

Yeast cultures were cultivated for 7 days in WMVIII. Then, the totality of the sterols was determined as described above. The free sterols are determined by GC from yeasts that are encapsulated with glass pearls and are extracted with n-hexane.

The results are shown in Table 5.

The results show that the enzyme sterol-acyl transferase (Sat1) esterifies with higher effectiveness in particular sterols that are lacking the 4,4-dimethyl group. Thus, a technical application for the separation of 4,-4-dimethylsterols from the corresponding demethylated forms is also suitable.

Table 5

Proportion by percentage of free sterols. Each sterol was determined as a free sterol (without solution) and was related to the total amount of this sterol. The absolute total sterol contents as area/g dry substance are indicated in parentheses. Lanosterol and 4,4-dimethylzymosterol are sterols with a 4,4-dimethyl group.

	% of free sterols	
	Control	AH22tH3ura8/pADL-SAT1
Lanosterol	54 (0.99)	59 (2.90)
4,4-dimethylzymosterol	58 (0.77)	84 (2.37)
4-methylzymosterol	7 (2.43)	10 (7.62)
zymosterol	10 (1.67	11 (5.85)
ergost-7-enol	24 (4.55)	12 (9.00)
ergosta-5,7-dienol		

Description of the Figures

Fig. 1 shows plasmid YEpH2 with the corresponding interfaces.

Fig. 2 shows the plasmid YDpUHK3 with the corresponding interfaces.

Fig. 3 shows the plasmid pADL-SAT1 with the corresponding interfaces.

Fig. 4 shows the growth behavior and ergosterol and squalene contents with different uracil supplementation. In the figure, OD = optical density, Kultivierungszeit = cultivation time, Hefe-Trockengewicht = yeast dry weight, Uracilsupplementation = uracil supplementation.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT

(A) NAME: Schering AG

(B) STREET: Müllerstrasse 178

(C) CITY: Berlin

(E) COUNTRY: Germany

(F) POSTAL CODE (ZIP): D-13342

(G) TELEPHONE: (030)-4681 2085

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(ii) TITLE OF INVENTION: PROCESS FOR THE PRODUCTION OF ERGOSTEROL AND ITS INTERMEDIATE PRODUCTS USING RECOMBINANT YEASTS

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Patentin Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 25 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) HYPOTHETICAL: NO

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

5′-ACTATGGACC AATTGGTGAA AACTG

(2) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) HYPOTHETICAL: NO

(iii) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

5′-AGTCACATGG TGCTGTTGTG CTT

(2) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) HYPOTHETICAL: NO

(iii) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

5′-GAATTCAACC ATGGACAAGA AGAAG

(2) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) HYPOTHETICAL: NO

(iii) SEQUENCE DESCRIPTION: SEQ ID NO 4:

5′-AGAATTCCAC AGAACAGTTG CAGG

Claims

1. Process for the production of ergosterol and its intermediate products, characterized in that

a) first a plasmid is designed, into which several suitable genes of the ergosterol metabolic process are inserted in altered form,

or

b) first plasmids are designed, into which in each case one of the genes of the ergosterol metabolic process is inserted in altered from,

c) microorganisms are transformed with the thus produced plasmids, whereby the microorganisms are transformed with a plasmid under a) or they are transformed simultaneously or in succession with several plasmids under b),

d) fermentation into ergosterol is performed with the thus produced microorganisms,

e) after fermentation has ended, the ergosterol and its intermediate products are extracted from the cells and analyzed, and finally,

f) the thus obtained ergosterol and its intermediate products are purified using column chromatography and isolated.

2. Process according to claim 1, wherein

a-i) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

ii) the gene of squalene synthetase (**ERG9**),

iii) the gene of Acyl-CoA: sterol-acyl transferase (**SAT1**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-ii) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

and

ii) the gene of squalene synthetase (**ERG9**),

or

a-iii) first a plasmid is designed, into which the following genes are inserted:

i) the gene of HMG-Co-A-reductase (t-**HMG**),

and

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**),

or

a-iv) first a plasmid is designed, into which the following genes are inserted:

i) the gene of the HMG-Co-A-reductase (t-**HMG**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-v) first a plasmid is designed, into which the following genes are inserted:

ii) the gene of squalene synthetase (**ERG9**),

and

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**)

or

a-vi) first a plasmid is designed, into which the following genes are inserted:

ii) the gene of squalene synthetase (**ERG9**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

a-vii) first a plasmid is designed, into which the following genes are inserted:

iii) the gene of acyl-CoA: sterol-acyl transferase (**SAT1**),

and

iv) the gene of squalene epoxidase (**ERG1**),

or

b) first plasmids are designed, into which in each case one of the genes that is mentioned under a-i) is inserted,

and

c) microorganisms are transformed with the thus produced plasmids, whereby the microorganisms are transformed with a plasmid under a-i) to a-vii), or they are transformed simultaneously or in succession with several plasmids under b),

d) fermentation into ergosterol is performed with the thus produced microorganisms,

e) after fermentation has ended, the ergosterol and its intermediate products are extracted from the cells and analyzed, and finally

f) the thus obtained ergosterol and its intermediate products are purified using column chromatography and isolated.

3. Process according to claim 2, wherein in addition the gene of squalene epoxidase (**ERG1**) is inserted into the plasmid under a-ii), a-iii) and a-v), and in addition the gene of the acyl-CoA: sterol-acyl transferase is inserted into plasmid a-ii).

4. Process for the production of ergosterol and its intermediate products, wherein the genes that are mentioned in claim 1 under a), those in claim 2 under a-i) to a-vii) and those in claim 3 under a-ii), a-iii) and a-v) in each case with the plasmids are first introduced independently of one another into microorganisms of the same species, and fermentation into ergosterol is performed with them together and the ergosterol that is thus obtained is extracted from the cells, analyzed and purified using column chromatography and isolated.

5. Process according to claims 1 to 4, wherein the intermediate products are squalene, farnesol, geraniol, lanosterol, zymosterol, 4,4-dimethylzymosterol, 4-methylzymosterol, ergost-7-enol and ergosta-5,7-dienol.

6. Process according to claims 1 to 4, wherein the intermediate products are sterols with 5,7-diene structure.

7. Process according to claims 1 to 4, wherein the plasmids are plasmids YEpH2, YDpUHK3 and pADL-SAT1.

8. Process according to claims 1 to 4, wherein the microorganisms are yeasts.

9. Process according to claim 8, wherein it is the species **S. cerevisiae.**

10. Process according to claim 9, wherein it is the strain **S. cerevisiae** AH22.

11. Yeast strain **S. cerevisiae** AH22 that contains one or more of the genes that are mentioned under a-i) in the process.

12. Plasmid YEpH2 that consists of the average **ADH**-promoter, t-HMG (altered variant of **HMG-1**) and the **TRP**-terminator (Fig. 1).

13. Plasmid YDpUHK3 that consists of the average **ADH**-promoter, t-HMG (altered variant of the **HMG-1**) and the **TRP**-terminator, the gene for the kanamycin resistance and the **ura3** gene (Fig. 2).

14. Plasmid pADL-SAT1 that consists of the **SAT1** gene and the **LEU2** gene of YEp13.

15. Use of the plasmids according to claims 12 to 14 for the production of ergosterol.

16. Use of the plasmids according to claims 12 to 14 for the production of ergosterol intermediate products squalene, farnesol, geraniol, lanosterol, zymosterol, 4,4-dimethylzymosterol, 4-methylzymosterol, ergost-7-enol and ergosta-5,7-dienol.

17. Use of the plasmids according to claims 12 to 14 for the production of sterols with 5,7-diene structure.

18. Expression cassette that comprises the average **ADH**-promoter, the **t-HMG** gene, the **TRP**-terminator and the **SAT1** gene with the average **ADH**-promoter and the **TRP**-terminator.

19. Expression cassette that comprises the average **ADH**-promoter, the **t-HMG** gene, the **TRP**-terminator, the **SAT1** gene with the average **ADH**-promoter and the **TRP**-terminator, and the **ERG9**-gene with the average **ADH**-promoter and the **TRP**-terminator.

20. Combination of expression cassettes, whereby the combination consists of

a) a first expression cassette, on which the **ADH**-promoter, the **t-HMG**-gene, and the **TRP**-terminator are located,

b) a second expression cassette, on which the **ADH**-promoter, the **SAT1**-gene and the **TRP**-terminator are located,

and

c) a third expression cassette, on which the **ADP**-promoter, and the **ERG9**-gene with the **TRP**-terminator are located.

21. Use of the expression cassettes according to claims 18 to 20, for the transformation of microorganisms, which are used in the fermentation into ergosterol.

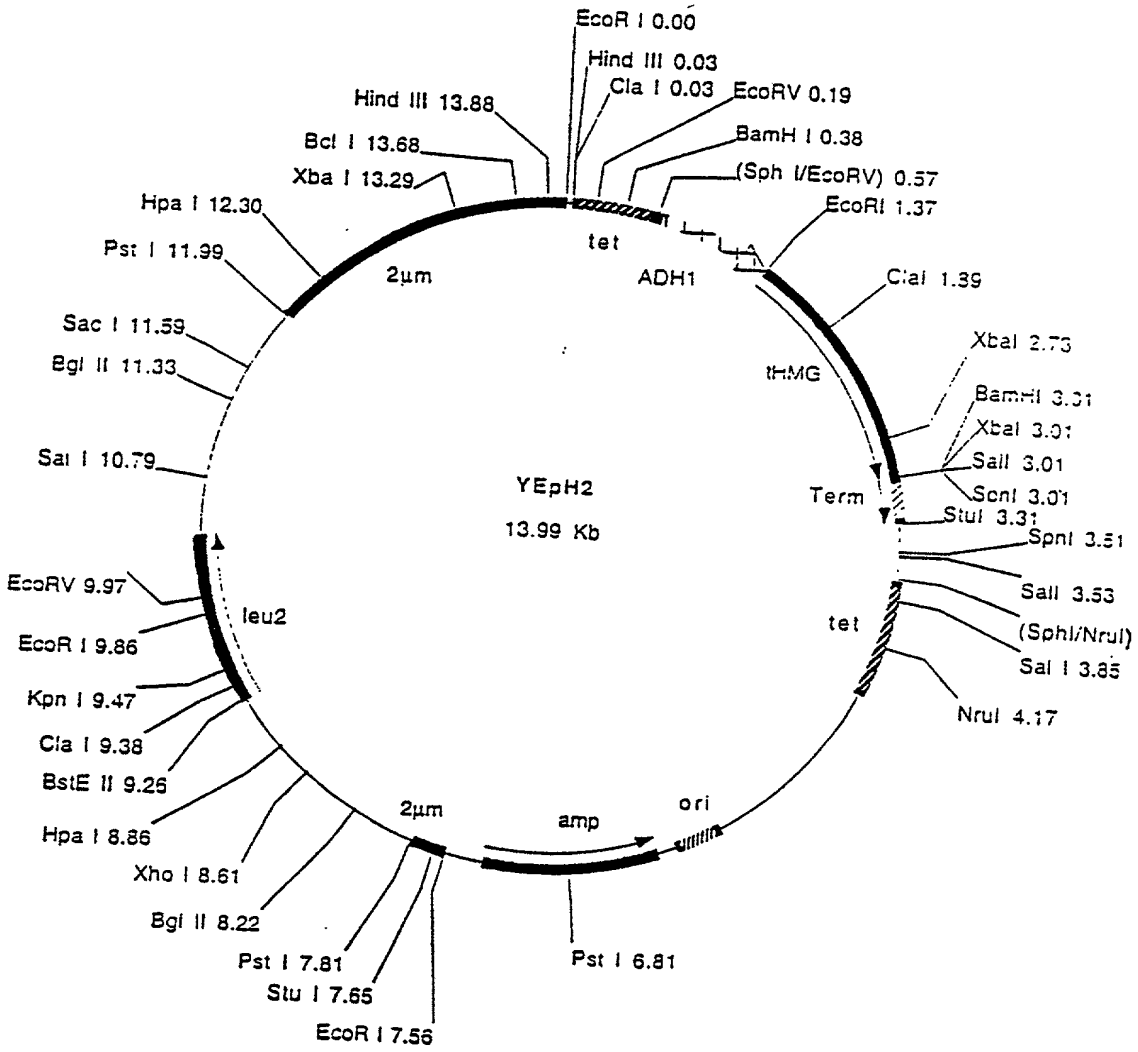
22. Use according to claim 21, wherein the microorganism is yeast.

23. Microorganisms that contain expression cassettes according to claims 18 to 20.

24. Microorganism according to claim 23, wherein it is yeast.

25. Use of the microorganism according to claims 23 and 24 in the fermentation into ergosterol.

26. Use of the microorganism according to claims 23 and 24, in the fermentation into ergosterol intermediate products.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Hind III 0.03
Cla I 0.03
EcoRV 0.19
BamH I 0.38
(Sph I/EcoRV) 0.57
EcoRI 1.37
ClaI 1.39
XbaI 2.73
BamHI 3.01
XbaI 3.01
SalI 3.01
SpnI 3.01
StuI 3.31
SpnI 3.51
SalI 3.53
(SphI/NruI)
Sal I 3.85
NruI 4.17
Pst I 6.81
Stu I 7.65
EcoR I 7.56
Pst I 7.81
Bgl II 8.22
Xho I 8.61
Hpa I 8.86
BstE II 9.26
Cla I 9.38
Kpn I 9.47
EcoR I 9.86
EcoRV 9.97
Sal I 10.79
Bgl II 11.33
Sac I 11.59
Pst I 11.99
Hpa I 12.30
Xba I 13.29
Bcl I 13.68
Hind III 13.88
YEpH2
13.99 Kb
2µm
tet
ADH1
tHMG
Term
tet
2µm
amp
ori
leu2

Fig. 1

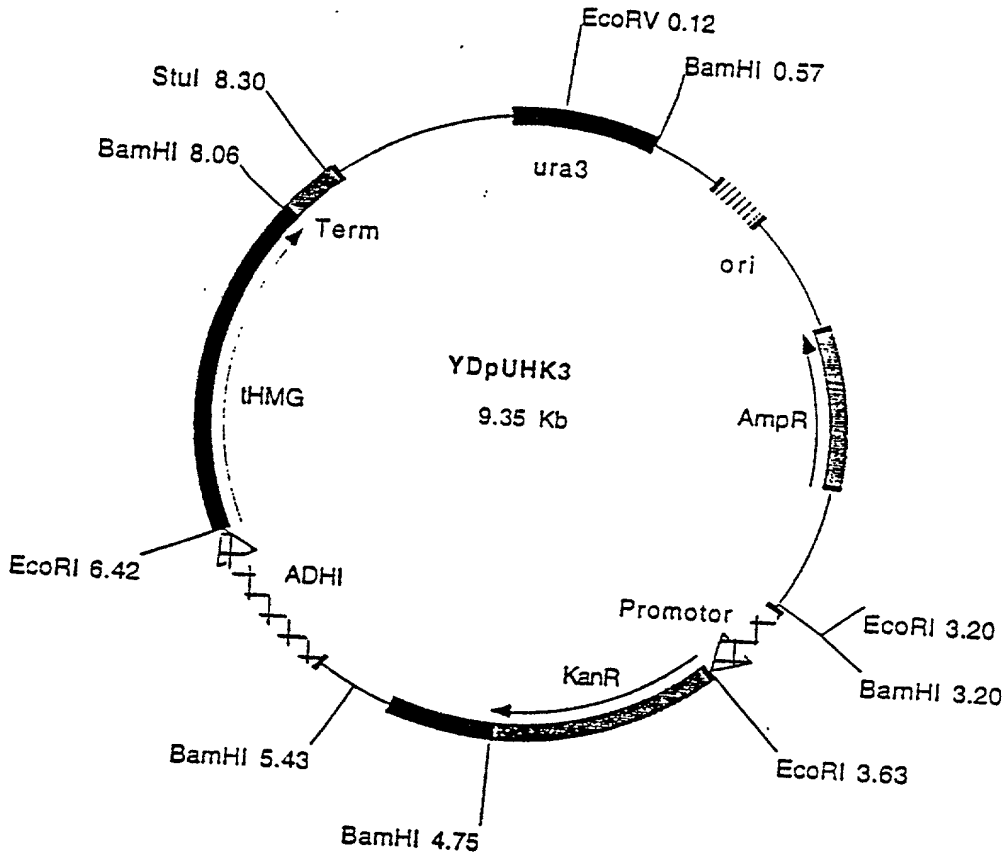
EcoRV 0.12
BamHI 0.57
StuI 8.30
BamHI 8.06
ura3
Term
ori
YDpUHK3
9.35 Kb
tHMG
AmpR
EcoRI 6.42
ADHI
Promotor
EcoRI 3.20
BamHI 3.20
KanR
BamHI 5.43
EcoRI 3.63
BamHI 4.75

Fig. 2

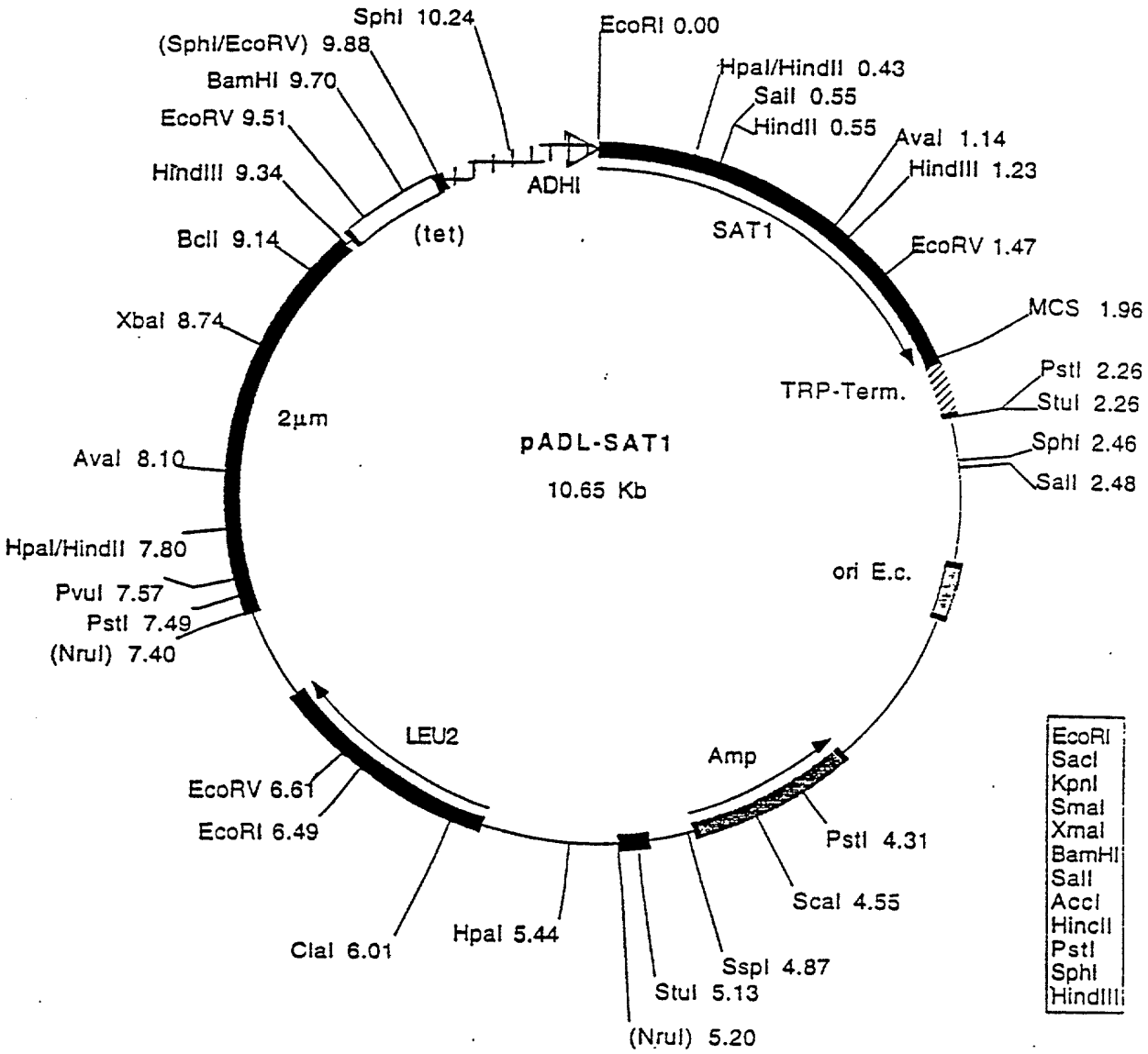
pADL-SAT1
10.65 Kb
EcoRI 0.00
HpaI/HindII 0.43
SalI 0.55
HindII 0.55
AvaI 1.14
HindIII 1.23
EcoRV 1.47
MCS 1.96
PstI 2.26
StuI 2.26
SphI 2.46
SalI 2.48
ori E.c.
Amp
PstI 4.31
ScaI 4.55
SspI 4.87
StuI 5.13
(NruI) 5.20
HpaI 5.44
ClaI 6.01
EcoRV 6.61
EcoRI 6.49
LEU2
(NruI) 7.40
PstI 7.49
PvuI 7.57
HpaI/HindII 7.80
AvaI 8.10
2µm
XbaI 8.74
BclI 9.14
HindIII 9.34
EcoRV 9.51
BamHI 9.70
(SphI/EcoRV) 9.88
SphI 10.24
(tet)
ADHI
SAT1
TRP-Term.
EcoRI
SacI
KpnI
SmaI
XmaI
BamHI
SalI
AccI
HincII
PstI
SphI
HindIII

Fig. 3

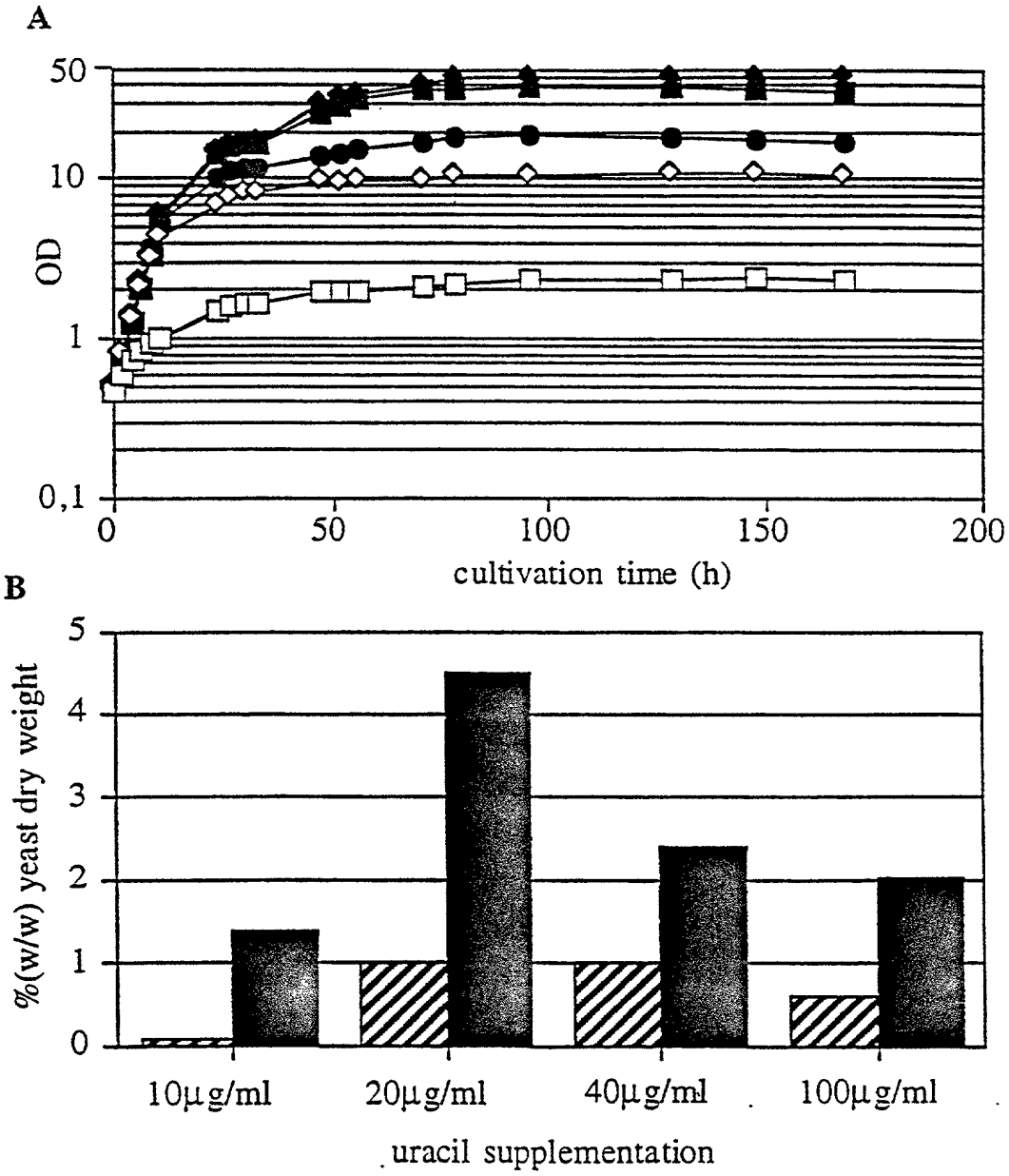
A
50
10
1
0,1
OD
0
50
100
150
200
cultivation time (h)
B
5
4
3
2
1
0
%(w/w) yeast dry weight
10µg/ml
20µg/ml
40µg/ml
100µg/ml
uracil supplementation

Fig. 4

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)	ATTORNEY'S DOCKET NUMBER SCH 1743

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought of the invention entitled.

PROCESS FOR THE PRODUCTION OF ERGOSTEROL AND ITS INTERMEDIATE PRODUCTS USING RECOMBINANT YEASTS

the specification of which (check only one item below)

- ☐ is attached hereto
- ☐ was filed as United States application

  Serial No. _____

  on _____

  and was amended

  on _____ _____ (if applicable)
- ☒ was filed as PCT international application

  Number PCT/EP98/06134 on 23 Septeber 1998

  and was amended under PCT Article 19

  on _____ _____ (if applicable)

☐ I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above

☐ I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim priority benefits under Title 35, United States Code, §119 of the following United States Provisional Application and of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR U.S. PROVISIONAL AND FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
Germany	197 44 212.9	30 September 1997	☒ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO

Combined Declaration For Patent Application and Power of Attorney (Continued)

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: SCH 1743

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			

POWER OF ATTORNEY: As a named inventor, I hereby appoint I. William Millen (19,544), John L. White (17,746), Anthony J. Zelano (27,969), Alan E.J. Branigan (20,565), John R. Moses (24,983), Harry B. Shubin (32,004), Brion P. Heaney (32,542), Richard J. Traverso (30,595), John A. Sopp (33,103), Richard M. Lebovitz (37,067), John H. Thomas (33,460), Catherine M. Joyce (40,668), James T. Moore (35,619), James E. Ruland (37,432), Nancy Axelrod (44,014) and Jennifer J. Branigan (40,921) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Combined Declaration for Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: SCH 1743

205	FULL NAME OF INVENTOR	FAMILY NAME: STAHL	FIRST GIVEN NAME: Ulf	SECOND GIVEN NAME:
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	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
208	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY
209	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	STREET	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

SIGNATURE OF INVENTOR 201: Ulf Stahl	DATE: 16/03/00	SIGNATURE OF INVENTOR 207	DATE
SIGNATURE OF INVENTOR 202	DATE	SIGNATURE OF INVENTOR 208	DATE
SIGNATURE OF INVENTOR 203	DATE	SIGNATURE OF INVENTOR 209	DATE
SIGNATURE OF INVENTOR 204	DATE	SIGNATURE OF INVENTOR 210	DATE
SIGNATURE OF INVENTOR 205	DATE	SIGNATURE OF INVENTOR 211	DATE
SIGNATURE OF INVENTOR 206	DATE	SIGNATURE OF INVENTOR 212	DATE

Combined Declaration for Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: SCH 1743

205	FULL NAME OF INVENTOR	FAMILY NAME: STAHL	FIRST GIVEN NAME: Ulf	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: Berlin	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: Muhlenfeldstrasse 115	CITY: Berlin	STATE & ZIP CODE/COUNTRY: D-13467 Germany
206	FULL NAME OF INVENTOR	FAMILY NAME: POLAKOWSKI	FIRST GIVEN NAME: Thomas	SECOND GIVEN NAME:
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	POST OFFICE ADDRESS	STREET: Egelsstrasse 2	CITY: Berlin	STATE & ZIP CODE/COUNTRY: D-13507 Germany
207	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
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	POST OFFICE ADDRESS	STREET:	CITY:	STATE & ZIP CODE/COUNTRY:
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	POST OFFICE ADDRESS	STREET:	CITY:	STATE & ZIP CODE/COUNTRY:
209	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
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SIGNATURE OF INVENTOR 201	DATE	SIGNATURE OF INVENTOR 207	DATE
SIGNATURE OF INVENTOR 202	DATE 22.03.2000	SIGNATURE OF INVENTOR 208	DATE
SIGNATURE OF INVENTOR 203	DATE 23.03.2000	SIGNATURE OF INVENTOR 209	DATE
SIGNATURE OF INVENTOR 204	DATE	SIGNATURE OF INVENTOR 210	DATE
SIGNATURE OF INVENTOR 205	DATE	SIGNATURE OF INVENTOR 211	DATE
SIGNATURE OF INVENTOR 206	DATE	SIGNATURE OF INVENTOR 212	DATE

Combined Declaration for Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER: SCH 1743

205	FULL NAME OF INVENTOR	FAMILY NAME: STAHL	FIRST GIVEN NAME: Ulf	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: Berlin	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: ~~Germany~~ Austrian
	POST OFFICE ADDRESS	STREET: Muhlenfeldstrasse 115	CITY: Berlin	STATE & ZIP CODE/COUNTRY: D-13467 Germany
206	FULL NAME OF INVENTOR	FAMILY NAME: POLAKOWSKI	FIRST GIVEN NAME: Thomas	SECOND GIVEN NAME:
	RESIDENCE & CITIZENSHIP	CITY: Berlin	STATE OR FOREIGN COUNTRY: Germany	COUNTRY OF CITIZENSHIP: Germany
	POST OFFICE ADDRESS	STREET: Egelsstrasse 2	CITY: Berlin	STATE & ZIP CODE/COUNTRY: D-13507 Germany
207	FULL NAME OF INVENTOR	FAMILY NAME:	FIRST GIVEN NAME:	SECOND GIVEN NAME:
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SIGNATURE OF INVENTOR 202	DATE	SIGNATURE OF INVENTOR 208	DATE
SIGNATURE OF INVENTOR 203	DATE	SIGNATURE OF INVENTOR 209	DATE
SIGNATURE OF INVENTOR 204	DATE 28.3.00	SIGNATURE OF INVENTOR 210	DATE
SIGNATURE OF INVENTOR 205	DATE 28.3.00	SIGNATURE OF INVENTOR 211	DATE
SIGNATURE OF INVENTOR 206	DATE 25.3.00	SIGNATURE OF INVENTOR 212	DATE